Journal of Chromatography B 1092 (2018) 7–14

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Heavy water enhancement of fluorescence signal in reversed-phase liquid chromatography

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ARTICLE INFO

Keywords:
Liquid chromatography
Heavy water
Fluorescence
Deuterium
Mass spectrometry
Doxorubicin
Fluorescein
Vitamin B_2

ABSTRACT

Liquid chromatography with fluorescence detection has been used in analyses demanding high sensitivity and selectivity. As majority systems rely on reversed-phase columns with water being the main component of the mobile phase, fluorescent compounds with emission maxima higher than 500 nm might be dynamically quenched. A simple replacement of H_2O with D_2O enhanced the sensitivity for selected compounds by 10–200%. Affected compounds included an anti-cancer drug doxorubicin, a luminescent probe fluorescein, and naturally occurring forms of vitamin B_2. Similar levels of enhancement were obtained by fluorescence spectrometry. Such simple yet effective approach may greatly improve HPLC analyses coupled to fluorescence detection.

1. Introduction

Fluorescence detection in liquid chromatography (LC) has been widely spread due to a number of key features, such as high sensitivity, high selectivity, and repeatability. Reversed-phase high-performance LC (RP HPLC) with the C_{18} modified silica has become the most frequent type of an HPLC column since the market introduction. There might be some drawbacks when using light water in RP HPLC with fluorescence detection as water molecules can cause numerous collisions with the excited fluorophore molecules, which result in a non-radiative relaxation. The process was described as luminescence dynamic quenching and the energy is dissipated as heat *via* the −OH oscillation [1]. Dynamic quenching is typical for luminophores with emission maxima higher than 500 nm as it is accompanied by relatively high absorbance of H_2O above 500 nm [2].

When the water is replaced with another solvent the fluorescence intensity, lifetime, as well as fluorescence quantum yield might change. For example, luminescence lifetime of ethidium bromide was 1.8 ns in H_2O but increased to 5.0 ns in DMSO, 6.9 in ethanol, 5.9 ns in glycerol, or to 6.3 ns in D_2O [3]. The shorter fluorescence lifetime of ethidium bromide in light water was attributed to the non-radiative deactivation process described above. The quantum yield of the fluorescent dye ATTO 655 (0.23 in H_2O) was increased to 0.39 in methanol, 0.55 in acetone, 0.56 in DMSO, and to 0.48 in D_2O [1].

Dynamic fluorescence quenching induced by deuterated water was found minimal in comparison to H_2O. One of the explanations relies on low absorbance of D_2O in a region above 500 nm [2]. Hence, if H_2O is replaced by D_2O in the mobile phase, the fluorescence signal-to-noise response may increase without any further modification of the compound separation. Although the enhancement induced by D_2O replacement is neither uniform nor known beforehand, an increase of fluorescence intensity and lifetime between units and hundreds of per cent might be observed.

We have selected a couple of compounds to demonstrate the aforementioned behavior of fluorophores surrounded by D_2O instead of H_2O. In order to enlighten the relationship between fluorescence intensity in light and heavy water, mass spectrometry was used as well.

Doxorubicin, which has been used as an anticancer agent, has been analyzed with satisfactory sensitivity in various biological fluids by chromatography [4–6] or targeted by fluorescence probes [7]. Fluorescein, which is heavily employed as a fluorescence labeling agent [8], has been used in imaging [9], chromatography [10,11], in cell targeting [12], or sensing [13]. Trace amounts of vitamin B_2 has been commonly determined by fluorescence techniques [14–17]. The mentioned applications may greatly benefit from the employment of heavy water into the methodology even as the overall impact and utilization still remain to be answered in future. In other words, we do not expect all researchers immediately exchanging their mobile phases, however; it may help in specialized types of applications or analyses where the limits of detection need to be pushed even further. It includes micro- or nano-HPLC to maintain the costs reasonable.

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https://doi.org/10.1016/j.jchromb.2018.05.037
Received 26 April 2018; Received in revised form 25 May 2018; Accepted 26 May 2018
Available online 26 May 2018

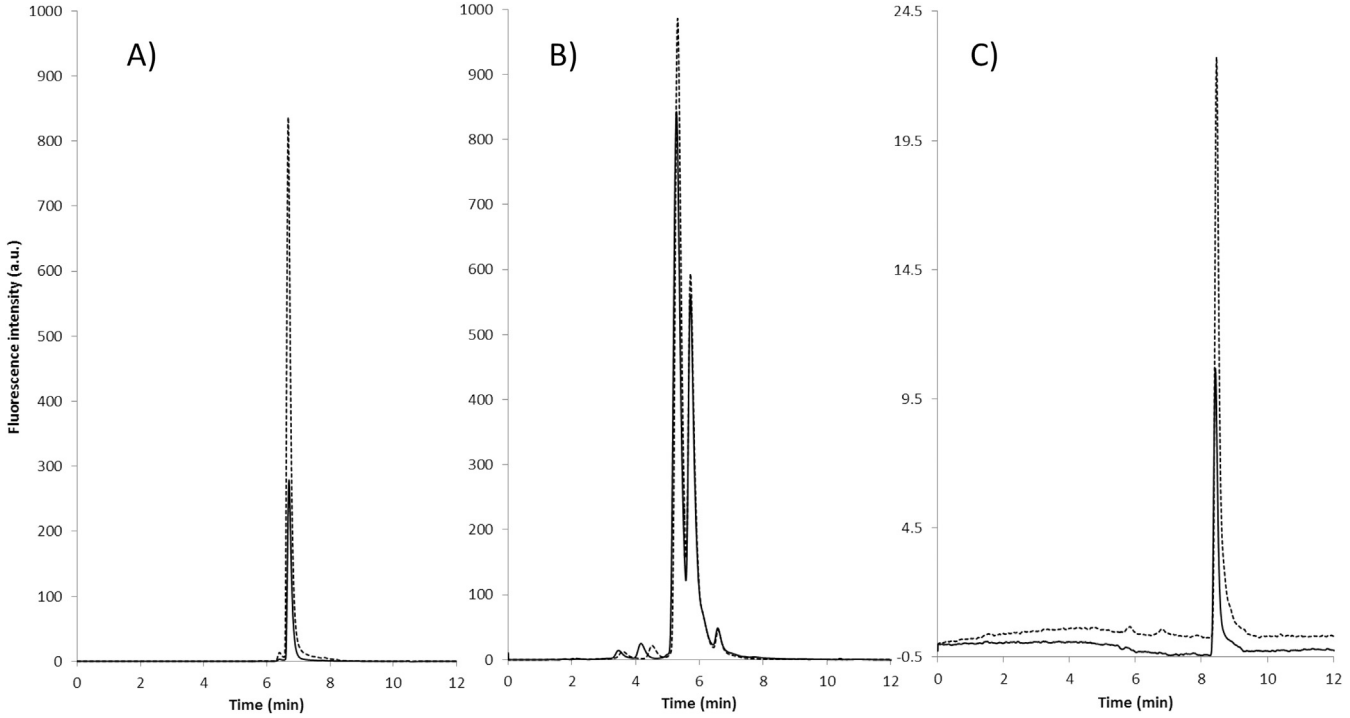

Fig. 1. Liquid chromatography with fluorescence detection of A) doxorubicin (26 μM, λ_{ex} = 480 nm, λ_{em} = 590 nm), B) vitamin B_2 species (15 μM each, (λ_{ex} = 377 nm, λ_{em} = 530 nm), and C) fluorscein (542 nM, λ_{ex} = 490 nm, λ_{em} = 518 nm) in H_2O (solid line) and D_2O (dashed line).

2. Experimental

2.1. Reagents and materials

Formic acid (LC MS), acetic acid (LC MS), ammonium hydroxide (28–30% NH_3 in water), sodium formate (LC-MS), acetonitrile (ACN, LC-MS), heavy water, fluorescein, doxorubicin, riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) were purchased from Sigma-Aldrich (Czech Republic). Light water was of an ultra-pure grade supplied by an in-house Milli-Q system (Millipore, MA, USA).

2.2. Fluorescence spectrometry

Stock solutions were prepared by dilution of solid chemicals in H_2O. The concentration of all stock solutions was 0.1 mM. Samples were prepared by dilution of the stock solutions in D_2O and H_2O. Final concentrations were 0.22 μM for fluorescein, 0.01 μM for doxorubicin, and 1.4 μM for FAD, FMN, and riboflavin. The pH (pD) value of sample solutions was checked to be 7 ± 0.05 and was not further adjusted.

A spectrofluorometer Aminco-Bowman Series 2 (Thermo Fisher Scientific, MA, USA) equipped with a 150 W Xe lamp was used for steady state measurements. Excitation and emission wavelengths were set at 445 nm and 525 nm for riboflavin, FMN, and FAD; 490 nm and 590 nm for doxorubicin; and 480 nm and 515 nm for fluorescein. The spectra were acquired with 1 nm resolution and a scanning speed of 1 nm/s.

Time-domain measurements were performed using TCSPC SPC-130EM (Becker and Hickl GmbH, Germany) instrumentation equipped with a 408 nm diode laser (picosecond resolution, repeating frequency 150 MHz). The emission monochromator was set at same values as in steady-state measurements. The quartz cuvettes were used in all experiments.

2.3. Liquid chromatography with fluorescence or mass spectrometric detection

Samples were measured on an LC system Shimadzu LC-10 ADVp (Shimadzu, Japan) consisting of a manual injector, low pressure gradient ternary pump, and a fluorescence detector RF 10AXL and a Dionex UltiMate 3000RS (Thermo Fisher Scientific, CA, USA) equipped with a high-pressure gradient binary pump, an autosampler, and a heated column compartment. The latter was connected to a Bruker MicrOTOF-Q II (Germany) mass spectrometer.

Compound separation was achieved with a 2.1 mm × 150 mm, 5 μm Acclaim 120 C_{18} (Thermo Fisher Scientific, CA, USA) column, and a flow-rate of 0.4 mL min^{-1}. The binary mobile phase system consisted of ACN and 0.1% formic acid for doxorubicin, ACN and 0.1% formic acid with 0.1% ammonia for B_2 vitamins, and pure H_2O without any additives for fluorescein analysis. H_2O or D_2O was used as a solvent for the mobile phase preparation. After a 20-μL injection, the percentage of ACN was linearly increased from 10% to 60% over 7 min where it was held for 2 min, followed by equilibration under the initial conditions for another 5 min. A complete LC run was 14 min.

Fluorescence detector was set at 480 nm excitation and 590 nm emission wavelengths for doxorubicin, at 377 nm excitation and 530 nm emission wavelengths for B_2 vitamins, and at 489 nm excitation and 512 nm emission wavelengths for fluorescein analysis.

MS was operated in the positive electrospray ionization mode and the ionization conditions were determined by the software as follows: capillary voltage: 4500 V, end plate offset: −500 V, source temperature: 220 °C, desolvation gas (nitrogen) flow: 8 L min^{-1}, nebulizer (nitrogen) pressure: 300 kPa, and collision cell voltage: 6 eV. The base peak chromatogram (BPC) was acquired in MS mode by monitoring the m/z range of 50–1500 with a spectra sample time of 1 s and MS/MS spectra were collected in a data-dependent mode. The mass spectrometer was calibrated using 10 mM sodium formate in 50% isopropyl alcohol on a daily basis and at the beginning of each LC run with an external 20-μL loop flush. High-resolution MS and MS/MS spectra were